Metabolomics to investigate the effects of treatments on food and of food consumption on health

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This PhD thesis aimed to advance the knowledge of the effects of treatment on food and food consumption on human health through metabolomics approaches. To this purpose, a specific SOP (Standard operation procedures) was set up and applied to deal with the metabolites of different food matrix. Then, metabolomics-oriented experiments focusing on the consequences of high hydrostatic pressure (HHP) on seafood products were carried out to investigate the effect of HHP on metabolic profile of seafood. Finally, a further metabolomics-oriented experiment targeted the potential mechanisms underlying the effect of a multistrain probiotic on cirrhotic patients.

La metabolomica per studiare gli effetti dei trattamenti sugli alimenti e del loro consumo sulla salute

Questa tesi di dottorato mirava a far progredire la conoscenza degli effetti del trattamento sugli alimenti e del loro consumo sulla salute umana attraverso approcci metabolomici. A tal fine, è stata messa a punto e applicata una specifica SOP (Standard operation procedures) per trattare i metaboliti di diverse matrici alimentari. In seguito, sono stati condotti esperimenti orientati alla metabolomica, incentrati sulle conseguenze dell'alta pressione idrostatica (HHP) sui prodotti ittici, per studiare l'effetto dell'HHP sul profilo metabolico dei frutti di mare. Infine, un ulteriore esperimento orientato alla metabolomica ha riguardato i potenziali meccanismi alla base dell'effetto di un probiotico multistrato sui pazienti cirrotici.

**Key words**: Metabolomics; High hydrostatic pressure; 1H-NMR, probiotic, human health.

# **1. Introduction**

Following the Ph.D. thesis project previously described (Lan, 2021), this oral communication reports the main results of the following four activities directed to:

A1) Literature review of the latest research related to investigating the effects of stress conditions on the physiological response and metabolism of food.

A2) Set up and application of specific NMR SOP to deal with the metabolites of seafood.

A3) Metabolomics-oriented experiments focus on the consequences of treatments on food composition and quality of treatments. The effects of HHP on the metabolism of grey mullet *(Mugil cephalus*), striped prawn *(Melicertus kerathurus*), and deep-water rose shrimp (*Parapenaeus longirostris*) during chilled storage were investigated.

A4) Metabolomics-oriented experiments focusing on the relationship between food composition and health.

# **2. Applications of metabolomics**

Metabolomics, the field of research dedicated to studying the complete collection of small metabolites in a biological system (known as the metabolome), has broad applications. In the context of food, metabolomics provides a systematic approach to identifying and quantifying its components. This enables us to understand the chemical and biochemical changes that occur due to technological processes or microbial activity. These changes ultimately determine important product characteristics, such as nutritional quality, safety, and sensory attributes. By monitoring changes in the entire food matrix, metabolomic approaches can provide valuable insights into the impact of various food traits and transformations on consumer acceptability. Moreover, since the composition of metabolites in food directly affects human health upon consumption, a comprehensive examination of food intake through metabolomic analysis can include studying the metabolite profile of body fluids (Trimigno, et al., 2020).

One of the preferred analytical platforms for investigating the metabolome of both food and human biofluids simultaneously is proton high-resolution nuclear magnetic resonance (1H-NMR). Its effectiveness in metabolomics research is evident in studies such as that of Yang et al. (2020), where they employed 1H-NMR to quantify taste-active metabolites and explore taste variations in different Chinese sauce-stewed beef. Similarly, Trimigno et al. (2020) utilized 1H-NMR spectroscopy to compare the metabolic effects of a nutritionally healthy New Nordic Diet with an Average Danish Diet by analyzing alterations in the human urine metabolome. Thus, 1H-NMR has emerged as a valuable tool for capturing information about changes in food quality resulting from various treatments, as well as understanding the connections between food composition and human health.

Regarding food processing, non-thermal technologies are gaining popularity in the field of food processing, particularly in developed countries. These technologies offer milder treatment conditions compared to heat exchange, resulting in higher quality while ensuring food safety. Among these technologies, high hydrostatic pressure (HHP) has been proven effective for preserving various seafood products by inhibiting the growth of undesirable spoilage microorganisms (Ekonomou & Boziaris, 2021). Metabolomics plays a crucial role in this context as it provides valuable insights into the consequences of microbial growth on sensory characteristics such as freshness and flavors, by tracking changes in the metabolome of fish flesh (Lou et al., 2020). For instance, the concentrations of adenosine triphosphate (ATP) and its breakdown products, including adenosine-5-diphosphate (ADP), adenosine-5-monophosphate (AMP), inosine-5-monophosphate (IMP), inosine, and hypoxanthine, serve as indicators of freshness in various seafood. Additionally, certain water-soluble, low-weight molecules are recognized as taste-active compounds contributing to specific flavors in seafood, categorized as umami, sweet, sour, and bitter (Nishimura & Kato, 1988). However, since the effects of HHP on seafood can vary and depend on both process parameters and seafood species (Puértolas & Lavilla, 2020), further research is necessary to gain a deeper understanding of how HHP affects specific seafood metabolomes.

The application of metabolomics in the study of probiotics and their impact on the human body in the context of nutrition is an intriguing research area. Probiotic bacteria are utilized in the production of functional foods to promote a healthy diet, leveraging their positive effects on the immune system and overall well-being. Remarkably, the mechanisms through which probiotics exert their health benefits remain largely unexplored. This holds true for various applications, including one examined by Román et al. (2019), who conducted a double-blind, placebo-controlled, randomized clinical trial to investigate the effects of a multistrain probiotic on individuals with cirrhosis.

# **2. Materials and Methods**

## **2.1 Effects of HHP treatment on the metabolic profile of seafood products**

**HHP treatment**

Striped prawns, rose shrimps, and grey mullets were fished in the Adriatic Sea. They were fast frozen at a temperature of -18°C for 24 h by the company Economia del Mare (Cesenatico, Italy). Seafood samples were subjected to mechanical deboning and shell removal after thawing at 4°C for 16 h. Flesh was manually cut into pieces and packed in polypropylene (PP) trays containing 6 monoportions of about 15-20 g each that were packed under vacuum with a PP film. Vacuum packed samples were subjected to HHP treatments (400, 500, and 600 MPa) for 10 min performed by the company HPP Italia s.r.l (Parma, Italy). The untreated sample was used as a control.

**Storage**

After treatment, samples were stored at 2±1°C and microbial shelf life ended when reaching a microbial load of 6 log CFU/g. During storage, samples were subjected to analytical determinations after 1, 6, 9, 14, 21, 28, and 35 days. For each HHP treatment and at each storage time, 3 different packages were used.

**Microorganisms analysis**

All the samples were investigated for the presence of *Salmonella spp*. and *Listeria monocytogenes* according to EN ISO 6579-1:2017/A1:2020 and ISO 11290-1:2017, respectively. Microbial groups considered in this research were total mesophilic bacteria (TMB), *Lactobacillus spp*., *Pseudomonas*, sulfite reducing anaerobic bacteria, total Coliforms, *E. coli*, and coagulase positive staphylococci.

**1H-NMR analysis**

A 1H-NMR analysis solution was prepared, constituted by a 10 mM D2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) as a chemical-shift reference (δ -0.017). A 1 M phosphate buffer granted a pH of 7.00 ± 0.02, while 10 μl of NaN3 (2 Mm) avoided microbial proliferation.

By modifying the procedure set up by Ciampa et al (2012). A trichloroacetic acid (TCA) extraction was performed, by adding 0.5 g of fish muscle to 3 mL of 7% (w/w) TCA, followed by homogenization by Ultra-Turrax (IKA, Germany) at 14,000 rpm for 20 s. The homogenate was centrifuged at 18630 g for 10 min at 4 °C, then 0.7 ml of supernatant was added with 0.100 ml of a D2O solution of 3-(trimethylsilyl)-propionic-2,2,3,3-d4 acid sodium salt (TSP) 10 mmol/L. The pH was adjusted to 7.00 ± 0.02 using 9 mol/L KOH in an Eppendorf microfuge tube. After centrifuging once more under the above conditions, 0.65 mL of supernatant was transferred to an NMR tube for analysis.

## **2.2 Effects of a multistrain probiotic on cirrhotic patients**

**Study Design**

Patients were randomized by a hepatologist, other than those who selected the patients, to take either a probiotic (probiotic group) or a placebo (placebo group). Randomization was performed by means of a computer‐generated sequence using blocks of four and consecutively numbered opaque sealed envelopes.32 patients were treated for 12 weeks and assessed at baseline and at 12 weeks (end of treatment) for clinical and analytical data, complications of cirrhosis, side effects, adherence, cognitive function, risk of falls, systemic inflammatory response, and biomarkers of intestinal barrier and bacterial translocation. There were no statistical differences between the 2 groups at baseline.

**1H-NMR analysis of serum**

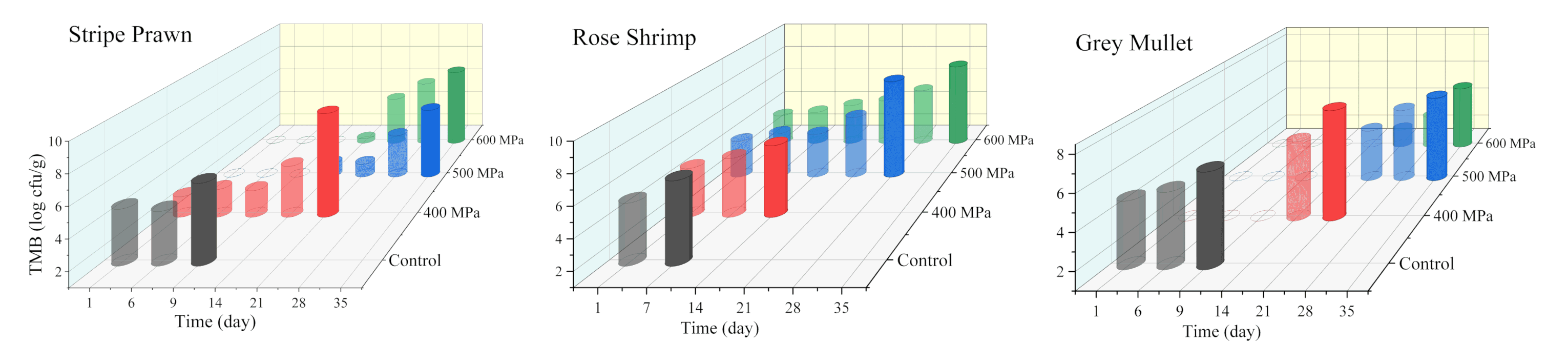
Serum samples were prepared for 1H-NMR by thawing and centrifuging 1 mL of each sample for 15 min at 18,630 g and 4°C. 500 μL of supernatant was added to 100 μL of NMR analysis solution. Urine samples were prepared for 1H-NMR by means of thawing and centrifuging them for 15 min at 18,630 g at 4°C. An amount of supernatant equal to 350 μL was added to 350 μL of bi-distilled water and to 200 μL of NMR analysis solution. Finally, each of the obtained samples was centrifuged again at the above conditions just before analysis.

# **3. Results and Discussion**

## **3.1 Results of HHP treatment on the metabolic profile of seafood products**

**Effect of HHP on microorganisms of considered seafood**

Figure 1 illustrates the results of the microorganism analysis. It is obvious that the application of 600 MPa pressure extended the microbiological shelf life of the seafood under consideration from 7 or 9 days to 30 days. All treated samples exhibited lower viable counts throughout the storage period compared to the untreated samples.



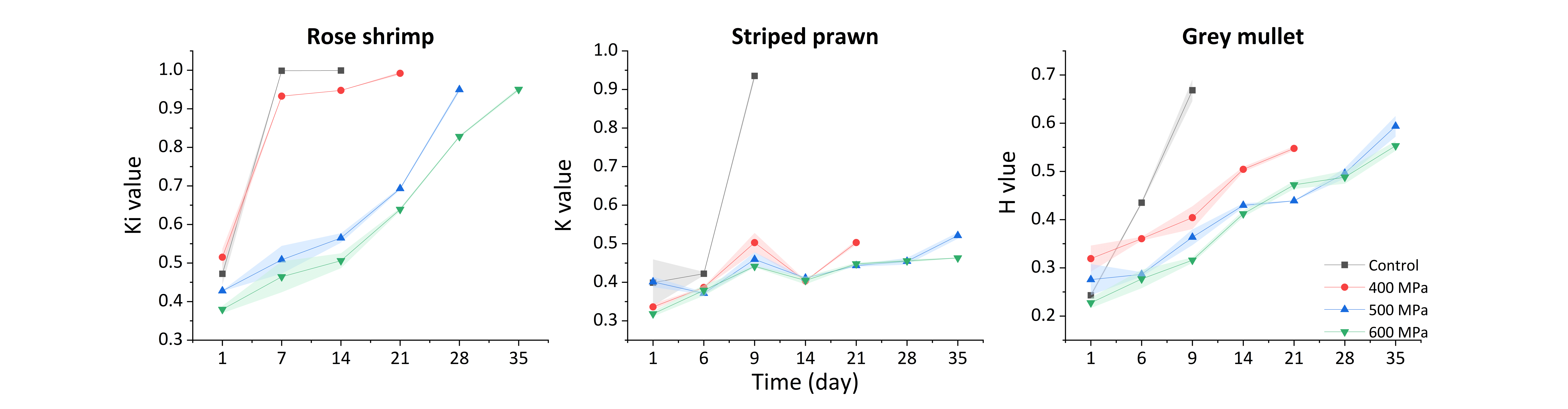
**Figure 1** *Changes in microbial cell loads (log CFU/g) of total mesophilic bacteria (TMB) of packaged striped prawn, rose shrimp, and grey mullet during the chilled storage of packaged striped prawn untreated (gray) or after treatment with HHP at 400 (red), 500 (blue) or 600 MPa (green).*

**Characterization of seafood metabolome**

1H-NMR spectra representative of the samples was shown in the Ph.D. thesis project previously described (Lan, 2022). The spectra of the considered seafood predominantly exhibit metabolite groups such as amino acids, amines, carbohydrates, nucleotides, and organic acids. Notably, there are notable differences in certain molecules between untreated and treated samples. Specifically, signals from specific molecules like putrescine and cadaverine, which are associated with spoilage, were only detected on the final day of storage. Additionally, the intensities of other signals, such as acetate, pyruvate, and threonine, displayed significant variations between treated and untreated samples.

**Effect of HHP on freshness-related metabolites during storage**

1H-NMR spectra made it possible to quantify the nucleotides used to calculate the freshness of the nucleotide breakdown of the seafood products considered. Based on the distinct nucleotide compositions detected by 1H-NMR in the three seafood (data not shown), Ki, K, and H values were employed to calculate the nucleotide breakdown freshness of rose shrimp, striped shrimp, and grey mullet, respectively. As depicted in Figure 2, the application of HHP treatment clearly exhibited a delay in nucleotide decomposition, effectively retarding the deterioration of freshness. Further analysis of the nucleotide concentrations during storage for each treatment (data not shown) suggests that HHP treatment impeded the conversion of IMP to inosine and/or inosine to hypoxanthine.



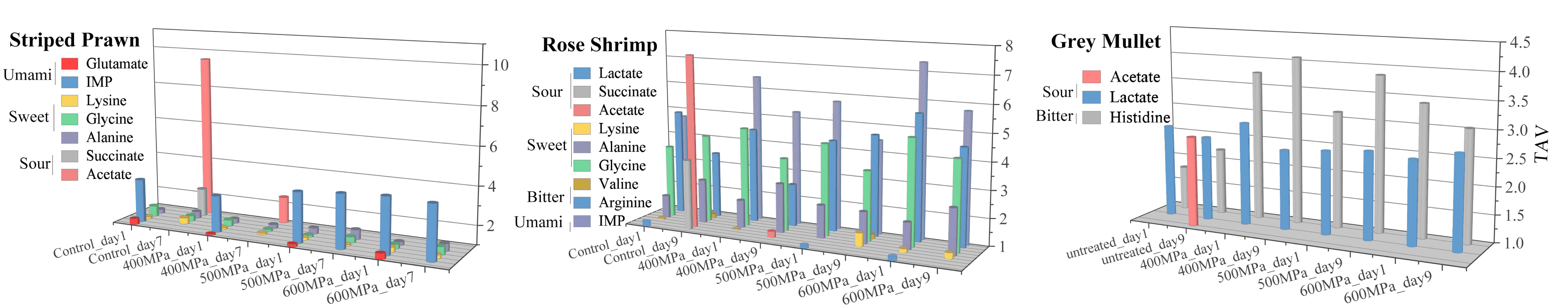
**Figure 2** *Changes of nucleotide degradation-related values of rose shrimp, striped prawn, and grey mullet untreated (black squares) and in samples treated with HHP at 400 (red circles), 500 (blue upward triangles), and 600 MPa (purple downward triangles). Ki value is the ratio of the total amount of inosine and hypoxanthine to that of IMP, inosine, and hypoxanthine. K value is the ratio of the total amount of inosine and hypoxanthine to that of all nucleotides. H value is the ratio of hypoxanthine to the total amount of IMP, inosine, and hypoxanthine.*

**Effect of HHP on TAV value of sensory active metabolites during storage**

To represent the contribution of the concentration of a taste-active compound to the sensory profile, its taste activity value (TAV) was calculated as the ratio of the compound concentration to its taste recognition threshold. In general, compounds with a taste activity value greater than 1 are considered as active compounds in food taste analysis (Figure 3).

Figure 3 illustrates the presence of various taste-active compounds in rose shrimp, striped prawn, and grey mullet on day 1. In rose shrimp, glutamate and IMP were detected at concentrations surpassing the threshold for umami taste. Additionally, lysine, glycine, and alanine were identified as significant contributors to the sweet taste profile of rose shrimp. In the case of striped prawn, lactate, IMP, and arginine exceeded the threshold concentrations for sour, umami, and bitter tastes, respectively. Moreover, glycine, lysine, and alanine were found to have concentrations above the threshold for sweet taste in striped prawn. Lastly, in grey mullet, histidine and lactate surpassed their respective thresholds for bitter and sour tastes.

Another intriguing observation was both HHP treatment and storage had an impact on the TAV of taste-active compounds. This was observed especially for the TAV of acetate, which tended to be above 1 in three untreated seafood after 7 or 9 days of storage. In contrast, in 500 and 600 MPa treated seafood samples, the concentrations of acetate remained below the threshold after 7 or 9 days. A similar result was observed in concentrations of succinate and of IMP in rose shrimp and striped prawn. Lower concentrations of acetate and succinate and higher concentrations of IMP in treated samples compared with this in untreated samples suggest that HHP can reduce the sour metabolism production of acetate and succinate and slow down the degradation of IMP with umami taste.

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**Figure 3** *Taste-active molecules with TAVs greater than 1 in untreated and treated (400, 500, 600 MPa) rose shrimp, striped prawn, and grey mullet on day 1 and day 7 or 9. Not showed represents TAV less than 1.*

## **3.2 Results of a multistrain probiotic on cirrhotic patients**

**Characterization of cirrhotic patients’ serum metabolome**

The 1H-NMR representative spectra of cirrhotic patients’ serum were shown in Figure 4. The untargeted analysis of the serum metabolome using 1H-NMR enabled the clear identification of 54 metabolites.



**Figure 4** *Samples of 1H-NMR spectra of serum of patients with cirrhosis.*

**Pairwise comparisons of metabolites in the probiotic group and in the placebo group**

Figure 5 showed the pairwise comparisons between values at baseline and at 12 weeks. The probiotic group exhibited a significant increase in glutamine (p=0.002, FDR p=0.007) and a decrease in glutamate (p=0.03, FDR p=0.03), resulting in an elevated glutamine/glutamate ratio (p=0.009, FDR p=0.01). Conversely, the placebo group showed an increase in glutamate concentration (p=0.01, FDR p=0.02) and a decrease in the glutamine/glutamate ratio (p=0.02, FDR p=0.03). No statistically significant alterations were observed in any of the other identified metabolites. These findings suggest that the administration of multispecies probiotics can influence glutamine/glutamate metabolism and enhance the capacity to detoxify ammonia.



**Figure 5** *Volcano plots showing the change between baseline and 12 weeks in all metabolites identified in the probiotic group and in the placebo group.*

# **4. Conclusions and Future Perspectives**

The present research achieved a better understanding of the effect of HHP treatment on the metabolism of seafood. In particular, the application of HHP could effectively extend the microbiological shelf life and delay the degradation of freshness-related and taste-related metabolites resulting in a higher quality associated with freshness and flavor. The great intriguing thing is obtaining information on the main freshness index as well as the molecules umami, sweet, sour, and bitter related in seafood. Moreover, the potential mechanism underlying the effect of a multistrain probiotic on patients with cirrhosis was revealed. The use of probiotics significantly decreased blood glutamate levels and increased the glutamine and glutamine/glutamate ratio. A possible mechanism is the multispecies probiotics influence glutamine/glutamate metabolism and improve the ability to detoxify ammonia.

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